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(54) Title: LACTAM-CONTAINING COMPOSITIONS AND METHODS USEFUL FOR THE EXTRACTION OF NUCLEIC ACIDS

(57) Abstract

This invention relates to novel methods for the extraction of nucleic acid. In particular methods are described for isolating nucleic acid from a sample containing a complex biological mixture of nucleic acid and non-nucleic acids wherein the sample is combined with an extraction solution comprising a lactam and then the nucleic acid material is isolated from the resulting combined solution. The resulting combined solution is mixed and becomes biphasic and the nucleic acid material is isolated form the aqueous phase by precipitation with ethanol. The lactam is preferably about 5 to about 70 % of the extraction solution and is most preferably 2-pyrrolidone, N-ethyl-2-pyrrolidone, N-cyclohexyl-2-pyrrolidone, N-docecyl-2-pyrrolidone, N-methyl-2-pyrrolidone, N-hydroxyethyl-2-pyrrolidone, N-methyl-2-piperidone, 2-ε-caprolactam, N-methyl-2-caprolactam, 2-piperidone or N-(4-hydroxybenzyl)pyrrolidone. Methods for selectively isolating DNA, ribosomal RNA and plasmid DNA are also disclosed.

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LACTAM-CONTAINING COMPOSITIONS AND METHODS
USEFUL FOR THE EXTRACTION OF NUCLEIC ACIDS

BACKGROUND OF THE INVENTION

1.   Field of the Invention

          This invention relates to compositions and assay
methods for the extraction and hybridization of nucleic acids.
In particular, this invention relates to compositions and
methods to extract nucleic acids from cells in complex
biological samples or specimens. The method is based on the
use of the chemical family of compounds termed lactams,
preferably pyrrolidones, which promote nucleic acid base
pairing, and which are effective in the extraction and
purification of nucleic acids.

2.   Brief Description of the Relevant Art

          Organic solvents such as phenol and chloroform are
traditionally used in techniques employed to isolate nucleic
acid from procaryotic and eucaryotic cells or from complex
biological samples. Nucleic acid isolations typically begin
with an enzymatic digest performed with proteases followed by
cell lysis using ionic detergents and then extraction with
phenol or a phenol/chloroform combination. The organic and
aqueous phases are separated and nucleic acid which has
partitioned into the aqueous phase is recovered by
precipitation with alcohol. However, phenol or a
phenol/chloroform mixture is corrosive to human skin and is
considered as hazardous waste which must be carefully handled
and properly discarded. Further, the extraction method is time
consuming and laborious. Marmur, _J. Mol. Biol._, _3_:208-218
(1961), describes the standard preparative procedure for
extraction and purification of intact high molecular weight DNA
from procaryotic organisms using enzymatic treatment, addition
of a detergent, and the use of an organic solvent such as
phenol or phenol/chloroform. Chirgwin et al., _Biochemistry_,

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18:5294-5299 (1979) described the isolation of intact RNA from tissues enriched in ribonuclease by homogenization in guanidine thiocyanate and 2-mercaptoethanol followed by ethanol precipitation or by sedimentation through cesium chloride.

5   Further, the use of chaotropic agents such as guanidine thiocyanate (GnSCN) are widely used to lyse and release nucleic acid from cells into solution, largely due to the fact that the chaotropic salts inhibit nucleases and proteases. However, it has proved difficult to isolate the
10   nucleic acids from these chaotropic salt solutions due to the incompatibility of the chaotropes with ionic detergents and the inability to easily partition the nucleic acid into an aqueous phase, given such high molar concentrations of salt used and the chaotropic behavior of the salts.

15   Nucleic acid hybridization is a known and documented method for identifying nucleic acids. Hybridization is based on base pairing of complementary nucleic acid strands. When single stranded nucleic acids are incubated in appropriate buffer solutions, complementary base sequences pair to form
20   double stranded stable molecules. The presence or absence of such pairing may be detected by several different methods well known in the art.

          Most hybridization assays previously described involve multiple steps such as the hybridization technique
25   described by Dunn & Hassell in Cell, Vol. 12, pages 23-36 (1977). Their assay is of the sandwich-type whereby a first hybridization occurs between a "target" nucleic acid and a "capture" nucleic acid probe which has been immobilized on a solid support. A second hybridization then follows where a
30   "signal" nucleic acid probe, typically labelled with a radioactive isotope, hybridizes to a different region of the immobilized target nucleic acid. The hybridization of the signal probe may then be detected by, for example, autoradiography.

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SUMMARY OF THE INVENTION

This invention relates to novel methods for the extraction and hybridization of nucleic acids. In particular, methods are described for isolating nucleic acid from a sample

5   containing a complex biological mixture of nucleic acid and non-nucleic acids wherein the sample is combined with an extraction solution comprising a lactam and then the nucleic acid material is isolated from the resulting combined solution. The resulting combined solution is further mixed and allowed to

10   become biphasic and the nucleic acid material is isolated from the aqueous phase. The lactam is preferably about 5 to about 70% of the extraction solution, and is most preferably 2-pyrrolidone, N-ethyl-2-pyrrolidone, N-cyclohexyl-2-pyrrolidone, N-dodecyl-2-pyrrolidone, N-methyl-2-pyrrolidone, N-

15   hydroxyethyl-2-pyrrolidone, N-methyl-2-piperidone, 2-ε-caprolactam, N-methyl-2-caprolactam, 2-piperidone (or 2-δ-valerolactam) or N-(4-hydroxybenzyl)pyrrolidone.

Also disclosed is a method for the selective extraction of nucleic acid from a complex biological sample

20   comprising lysing the sample in a guanidine-thiocyanate buffer; combining the sample with an extraction solution containing a lactam and phenol to create a first aqueous phase and a first organic phase; and isolating DNA from the first aqueous phase. Ribosomal RNA (rRNA) may further be isolated from the first

25   organic phase by adding a buffer solution to the first organic phase, heating the first organic phase and buffer solution mixture to create a second organic phase and a second aqueous phase, and extracting rRNA from the aqueous phase.

Further disclosed is a method for the selective

30   extraction of plasmid DNA from a complex biological sample comprising partially solubilizing cells in the sample in a weak chaotropic salt solution such as one containing guanidine hydrochloride (GnHCl), removing insoluble material from the solution and then extracting plasmid DNA from the solution.

35   Additionally, hybridization assays are disclosed wherein a sample containing a complex biological mixture of nucleic acids and non-nucleic acids is combined with an extraction solution comprising a lactam, isolating nucleic acid

from the resulting combined solution and then a hybridization
assay is conducted with the isolated nucleic acid.

    The lactams used in the present invention are of a
very low order toxicity and are not corrosive to human tissue.
5   They retain most of the solvent properties of phenol or
phenol/chloroform and are uniquely suited to the application of
nucleic acid isolation and/or fractionation of biological
macromolecular complexes. In addition, they are substantially
less expensive than the commonly employed organic solvents.
10 Extraction by cesium chloride and alcohol precipitation may
also be avoided through the use of the methods of the present
invention, thus eliminating exposure to toxic cesium chloride.
The extraction methods of the present invention are faster,
simpler, safer and more sensitive than methods previously used
15 in the extraction of nucleic acid. Thus, they represent a
significant advance in the field, providing a means for scaling
up the extraction of desired nucleic acids.

    The use of lactams avoids problems relating to the
incompatibility of chaotropes with ionic detergents during
20 isolation of nucleic acids from chaotropic salt solutions. The
use of lactams also avoids difficulties arising during the
partitioning of nucleic acid into an aqueous phase, given the
high molar concentrations of salt typically used and the
chaotropic behavior of these salts. The use of lactams in
25 extraction techniques involving chaotropic salts allows the
rapid and simple recovery of intact RNA, DNA, or total nucleic
acid.

    An alternative method is also disclosed which allows
the extraction of nucleic acid without using heat. This method
30 provides for the extraction of nucleic acid from a complex
biological sample by combining the sample with an extraction
solution containing a guanidine salt, then adding a component
selected from the group consisting of alcohols, polysaccharide
sulfates and polymeric sulfonic acids, rendering the solution
35 biphasic and isolating the nucleic acid from the aqueous phase.

DESCRIPTION OF THE FIGURES
Figs. 1A and 1B illustrate various known synthetic pathways for the production of lactams.

DETAILED DESCRIPTION
5   This invention relates to novel means and methods for the extraction of nucleic acid from a complex biological mixture containing nucleic acids and non-nucleic acids. Highly sensitive hybridization assays are also described. The methods
10   of the present invention enable one to easily process a biological sample containing nucleic acids by promoting the lysis of cells in the sample and promoting the extraction of nucleic acids being present in the cells. The methods of the present invention further enable one to readily assay for a
15   nucleic acid suspected of being present in cells, i.e., a target nucleic acid. The extraction methods include lysing the cells in a solution comprising a lactam and then isolating the nucleic acid.

          The extraction and hybridization methods of the
20   present invention may be applied to a complex biological mixture of nucleic acid (RNA and/or DNA) and non-nucleic acid. Such a complex biological mixture includes a wide range of eucaryotic and procaryotic cells, including protoplasts; or other biological materials which may harbor nucleic acids. The
25   methods are thus applicable to tissue culture animal cells, animal tissue (e.g., heart, liver or brain, homogenized in lysis buffer), blood cells, reticulocytes, lymphocytes, plant cells or other cells sensitive to osmotic shock and cells of bacteria, yeasts, viruses, mycoplasmas, protozoa, rickettsia,
30   fungi and other small microbial cells and the like.

          The assay and isolation procedures of the present invention are useful, for instance, for detecting non-pathogenic or pathogenic microorganisms of interest. By extracting nucleic acid and detecting specific hybridization
35   between nucleotide probes of a known source and nucleic acids resident in the biological sample, the presence of the microorganisms may be established. Biological samples of

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interest could include virtually any type of specimen which may contain microorganisms of interest.

The nucleic acid sample to be assayed or to serve as a source for the extraction of nucleic acids is combined with
5   an extraction solution comprising a lactam. Lactams are a class of a group of organic cyclic compounds containing the -NH-CO- group in the ring. Such compounds are typically formed by the elimination of water from the amino and carboxyl groups of a noncyclic compound. The particular substituents on the
10   lactam ring are not critical to this invention. It is, however, important that the lactam maintain hydrogen bonding capacity, water solubility and the ability to form biphasic solutions upon heating or the addition of appropriate organic molecules. Thus, substituents of more than eight carbons, for
15   example, would be less preferred than substituents with less than eight carbons which would be more water soluble. This is especially true if the preferred substituents contain additional polar groups.

Preferably, the lactam will be of the general formula
20   including racemic mixtures and optically active isomers:
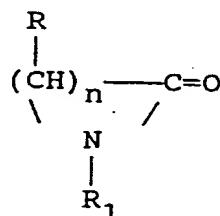
$$\begin{array}{c} R \\ | \\ (CH)_n \!\!-\!\!-\!\! C\!\!=\!\!O \\ \backslash \qquad / \\ N \\ | \\ R_1 \end{array}$$

25

30   wherein n = 3 to 8 (with the total number of R substituents being 3 to 8), and R_1 is selected from the group consisting of:
hydrogen,
alkyl of 1 to 20 carbons,
aryl of 6 to 10 carbons,
35   arylalkyl of 7 to 20 carbons,
alkylcarboxyamide of 1 to 20 carbons,
hydroxyarylalkyl of 7 to 20 carbons,
N-mono-substituted alkylcarboxyamide,
N, N-dialkyl-substituted alkylcarboxyamide,
40   alkylcarboxylate of 2 to 20 carbons,
acyl of 2 to 20 carbons,

cycloalkane of 4 to 20 carbons,

hydroxyalkyl,

and cyanoalkyl;

and where the R substituents can be the same or different and
selected from a group consisting of hydrogen, halogen and alkyl
of 1 to 5 carbons such that the total carbon number for all R
substituents does not exceed 20; with the further provision
that the total carbon number of the R_1 substituent does not
exceed 20 and where at least one R designated substituent is
hydrogen.

Pyrrolidones, piperidones and caprolactams are
lactams which are particularly preferred. The lactams which
are preferred also include those where n = 3 to 6; where at
least one R substituent is methyl; where the R_1 substituent and
all of the R substituents are hydrogen; where the R_1
substituent is other than hydrogen and the R substituents are
both hydrogen; where the R_1 substituent is a cycloalkane of 4
to 7 carbons; where the R_1 substituent is $-CH_2-CH_2OH$; where the
R_1 substituent is $-CH_2-CH_3$; where the R_1 substituent is methyl;
or where the R_1 substituent is dodecyl. Examples of these
preferred lactams which are most preferred are 2-pyrrolidone,
N-methyl-2-pyrrolidone, N-hydroxyethyl-2-pyrrolidone, N-
cyclohexyl-2-pyrrolidone, N-dodecyl-2-pyrrolidone, N-ethyl-2-
pyrrolidone, N-methyl-2-piperidone, 2-ε-caprolactam, N-methyl-
2-caprolactam, N-(4-hydroxybenzyl)pyrrolidone and 2-piperidone
(or 2-δ valerolactam). These are commercially available from
the GAF Chemicals Corporation (a subsidiary of GAF Corporation,
Wayne, New Jersey) and/or Aldrich Chemical Company (Milwaukee,
Wisconsin).

Alkyl refers to an aliphatic hydrocarbon radical,
$-(CH_2)_nCH_3$, either branched or unbranched such as methyl,
ethyl, n-propyl, isopropyl, n-butyl, isobutyl, sec-butyl,
tert-butyl, dodecyl or the like.

Aryl refers to a radical derived from an aromatic
hydrocarbon by removal of one hydrogen atom such as phenyl,
α-napthyl, ß-napthyl, biphenyl, anthryl and the like.

Arylalkyl, $-(CH_2)_n-Ar$, refers to an alkyl radical as
defined above joined to an aryl radical.

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Alkylcarboxyamide refers to a radical, $-(CH_2)_n-CONH_2$.

Hydroxyarylalkyl refers to an arylalkyl radical where the aryl radical is an hydroxyaryl.

N-mono-substituted alkylcarboxyamide refers to a radical,

$$-(CH_2)_n-\overset{\overset{\textstyle O}{\|}}{C}-NH(CH_2)_m-CH_3,$$ where n = 10 to 20 carbons and m = 1 to 5 carbons.

N,N-dialkyl-substituted alkylcarboxyamide refers to a radical,

$$-(CH_2)_n-\overset{\overset{\textstyle O}{\|}}{C}-N\overset{\displaystyle /(CH_2)_m-CH_3}{\displaystyle \backslash (CH_2)_m-CH_3}$$
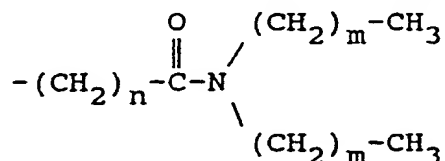
where each alkyl can be the same or different and where n = 10 to 20 carbons and m = 1 to 5 carbons.

Alkylcarboxylate refers to a radical $-(CH_2)_nCOO^-$, where n preferably = 1 to 19 carbons.

Acyl includes any organic radical derived from an organic acid, such as a carboxylic acid by elimination of the hydroxyl group. It is represented by the formula R_6-CO, wherein R_6 can be as defined in U.S. Patent No. 4,665,067 at column 2, line 31 through column 12, line 32, which is incorporated by reference herein. It is preferred that R_6 be an alkyl of 1 to 20 carbons or a cycloalkyl.

Hydroxyalkyl refers to a radical $-(CH_2)_nOH$, where n = 1 to 20.

Cycloalkane or cycloalkyl refers to a radical of a saturated hydrocarbon in a ring structure such as cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl, cycloheptyl, adamantyl and the like.

Cyanoalkyl refers to a radical of a cyano group, having the formula $-C\equiv N$ above.

Unless otherwise stated, all number ranges are inclusive of the stated range (e.g., 1 to 5 carbons includes 1 and 5 carbons).

Halogen refers to chlorine, bromine, fluorine and iodine.

Various pyrrolidone derivatives may be prepared as described in Kirk-Othmer Encyclopedia of Chemical Technology,

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19:510-518 (1982), incorporated by reference herein, or as
follows:

Alkali metal derivatives of pyrrolidone are readily
formed by reaction with metal or from concentrated caustic soda

5 and 2-pyrrolidone in acetone solution as described in
"Acetylene Chemistry," J.W. Reppe P.B. Report, 18-852-s, C.A.
Meyer & Co., Inc., New York, NY (1949), 210 pp., incorporated
by reference herein. Such procedure permits reaction with
substituted alkyl, aryl or arylalkyl halides without opening

10 the lactam ring, to give N-alkyl, N-aryl or N-arylalkyl
pyrrolidones. See Method A on Figure 1A. The latter two can
be nitrated without rupture of the lactam ring and alkyl,
hydroxy, alkoxy, or halogen substitution is also possible. N-
octyl-2-pyrrolidone and N-dodecyl-2-pyrrolidone are synthesized

15 in this manner and belong to the N-alkyl-2-pyrrolidone family.
N-methyl-2-pyrrolidone, the lactam of 4-methylaminobutyric
acid, is synthesized using the Reppe chemistry described above.
Acylated lactams can also be chlorinated and brominated at
elevated temperatures under the influence of light.

20 Lactams can react with the following reagents:
a) Acid anhydrides or chlorides result in formation
of N-acyl lactams; for example acetic anhydride forms from N-
acetyl-2-pyrrolidone. See Method B on Fig. 1A.
b) N-methylol-2-pyrrolidone results from reaction

25 of formaldehyde and 2-pyrrolidone.
c) N-(2-hydroxymethyl)-2-pyrrolidone is formed by
reaction with ethylene oxide. See Method C on Fig. 1A.
Other examples of various synthesis routes are also
set forth on Figure 1. Method D on Fig. 1A shows the reaction

30 of butyrolactone in the presence of a moderate excess of
ammonia under heat to yield 2-pyrrolidone. Method F on Fig. 1B
shows the same reaction using butyrolactone and alkylamine as
the starting materials to yield an alkyl substituted lactam.
Method E on Fig. 1A shows that lactams are easily

35 produced from the pyrolysis of amino acids. The reaction of
hydrazoic acids with cyclic ketones yield the corresponding
lactam as shown in method G on Fig. 1B. The commercially

available technical grades of all pyrrolidones should be vacuum
distilled prior to use with biological samples.

     The lactams should be provided in concentrations in
excess of about 5% of the total volume of the extraction
5   solution up to a concentration of about 70%, preferably a
concentration of 20% to 50%, most preferably a concentration of
about 30% to 50%. The lactams may be used singly or in
combination.

     Traditionally, organic solvents, such as phenol or a
10   phenol-chloroform combination are used to extract nucleic acid,
using a phase separation. These methods may be used
effectively with the extraction solutions of the present
invention; however, an advantage of the methods of the present
invention is that such toxic and tedious extraction methods are
15   not necessary.

Extraction of Total Nucleic Acid.

     The lactam-containing extraction solutions of the
present invention permit the complete extraction of total
20   nucleic acid (DNA and RNA). The lactam-containing extraction
solution is combined with the complex biological mixture so
that the combined solution becomes biphasic with the non-
nucleic acid material present in the organic phase and the
nucleic acid solution present in the aqueous phase. The
25   combined solution is typically mixed and subjected to
centrifugation and the nucleic acid in the aqueous phase is
precipitated with ethanol.

     The lactam-containing extraction solution will become
biphasic upon mixing with the nucleic acid sample. If the
30   lactam is miscible with water, such as N-hydroxyethyl-2-
pyrrolidone, then a biphasic state may be created upon mixing
the extraction solution with the nucleic acid sample and
heating the combined solution, typically to a temperature
greater than 40°C, usually to a temperature of about 45°C,
35   until the solution becomes biphasic. If the lactam is
immiscible with water, such as N-dodecyl-2- pyrrolidone or N-
octyl-2-pyrrolidone, then a biphasic state is created without
heat. Addition of other organic solvents, such as phenol, to

the extraction solution will also create a biphasic state for
extraction.   The extraction solution will typically be combined
with the nucleic acid sample so that the concentration of
lactam (or lactam and organic solvent) in the biphasic solution
5   is about 30-50%.

The extraction procedure will preferably use a
complex biological sample of a concentration such that after
combining the sample with the extraction solution, the amount
of nucleic acid present in the aqueous phase will not be in
10   excess of 1 mg/ml or such that the total cellular material does
not exceed 50 mg/ml.

Preferably, the extraction solution will contain
standard buffers and detergents to promote lysing of cells. A
buffer such as sodium citrate, Tris HCl, PIPES or HEPES,
15   preferably Tris-HCl at a concentration of about 0.05 to 0.1M
can be used. The extraction solution will typically also
contain about 0.05 to 0.5% of an ionic or nonionic detergent,
such as sodium dodecylsulfate (SDS) or Sarkosyl (Sigma Chemical
Co., St. Louis, Missouri) and between 1 to 10 mM EDTA.
20   Chaotropic agents which disturb the secondary and
tertiary structure of proteins, for example, guanidine salts
such as guanidine hydrochloride (GnHCl) and thiocyanate, or
urea, lithium chloride and other thiocyanates may be used in
combination with the lactams to dissociate nucleic acids and
25   inhibit nucleases. The use of chaotropic agents in the
extraction and hybridization of nucleic acids is described in
E.P. Publication No. 0 127 327, which is incorporated by
reference herein.

Extraction of total nucleic acid without the use of
30   heat may alternatively be performed by using a guanidine-based
chaotrope such as GnHCl which does not contain the SCN anion.
The complex biological sample may be lysed first with an
extraction solution comprising GnHCl and then contacted with an
extraction solution comprising a lactam, preferably N-methyl-
35   2-pyrrolidone. The extraction solution containing the lactam
may then be made biphasic by the addition of an organic solvent
such as phenol or chloroform. Total nucleic acid is isolated
from the aqueous phase.

Lactam-free Extraction Without Heat.

An alternative effective approach to using lactams in
extracting total nucleic acid from complex biological samples,
5   which surprisingly may be accomplished without heat is the use
of certain competitive agents from the group consisting of
alcohol, polysaccharide sulfate, and polymeric sulfonic acid in
conjunction with guanidine salt lysing agents. After
solubilizing the complex biological sample with a solution
10   comprising the guanidine salt, the competitive agent is added
at a molarity approximately comparable to or in excess of the
guanidine salt. The extraction solution is made biphasic by
the addition of an organic solvent such as lactam, phenol or
chloroform and nucleic acid is isolated from the aqueous phase.
15   No heat needs to be used in this extraction process. This
process is particularly beneficial when GnSCN is used as the
lysing agent.

The competitive agent alcohols are selected from the
group consisting of monohydroxyalkanes, dihydroxyalkanes,
20   trihydroxyalkanes with a total carbon number not exceeding 20,
monosaccharides, oligosaccharides, polysaccharides,
monohydroxycycloalkanes, dihydroxycycloalkanes,
trihydroxycycloalkanes and polyhydroxycycloalkanes. Preferably
the alcohol will be a dihydroxyalkane, most preferably ethylene
25   glycol. The competitive agent polysaccharide sulfates will
preferably be salts of polysaccharide chains of about 1,500 to
15,000 daltons, most preferably dextran sulfate.

The competitive agent polymeric sulfonic acid is a
sulfonic acid ranging from about 10,000 to 500,000 daltons.
30   Preferably the polymeric sulfonic acid is one which has
subunits having the general formula:

- (M) -   ; where M is a backbone component; and

35   X   X is a spacer group.

SO_3H
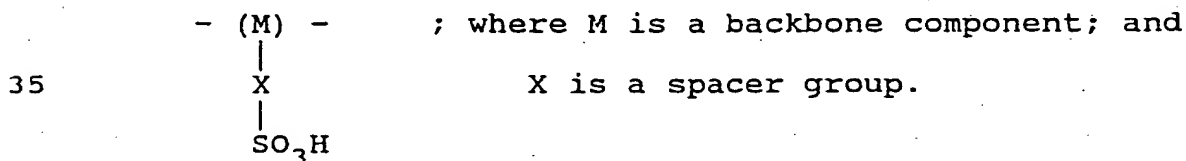
The backbone may comprise linear monomers, ringed monomers,
40   branched monomers or a combination of these monomers which are

linked together into a polymer. The term monomer is used here
to refer to a subunit, and does not imply that the monomer
necessarily be polymerizable with the spacer and sulfate group
in the final form present in the polymer. Appropriate

5   backbones, often substituted, include polyethylene,
polyacrylate chains, polyamide chains, polyacrylamide,
polypeptides, or polysaccharides.

     The hydrocarbon polymer backbone will preferably be
linear, but may contain branched structures. Polymer lengths

10   in the range of 1,500 to 20,000 daltons are preferred.
Polymers may be primarily homopolymers, and these homopolymers
might be linked together by identical (e.g. head to tail) or
nonidentical (e.g. head to head and tail to tail) linkages.
Alternatively, the polymers may comprise heterogenous subunits,

15   or be composed of combinations of various different types of
subunits (e.g. copolymers), in an ordered or random sequence.
The spacers will be hydrocarbon moieties. Typical spacers
include saturated or unsaturated alkyl groups, e.g., methylene,
ethylene and other alkyl groups, substituted phenyl groups,

20   e.g., 1,2 (ortho), 1,3 (meta) or 1,4 (para), benzyl groups, or
naphthyl groups. The spacers can be linear, branched or ringed
structures.

     Polyvinylsulfonic acid (PVSA) and polystyrene
sulfonic acid (PSSA) are the polymeric sulfonic acids most

25   preferred.

Sequential Selective Extraction of DNA and rRNA.

     Extraction solutions of the present invention also
permit the selective and sequential extraction of DNA and rRNA.

30   A complex biological sample is first lysed in a guanidine
thiocyanate buffer solution, such as a 3M GnSCN solution. The
lysed sample is then combined with an extraction solution
containing a lactam and phenol to create a first aqueous phase
and a first organic phase. A room temperature extraction is

35   performed where DNA is isolated from the first aqueous phase,
the rRNA remaining in the first organic phase. The rRNA is
isolated from the first organic phase after a standard buffer
solution, such as 1% SDS, 50 mM Tris, 25 mM EDTA and 0.05M

NaCl. This solution is heated, typically to about 65°C, to
create a second organic phase and a second aqueous phase. The
rRNA is then isolated from the second aqueous phase.

For the sequential DNA-rRNA isolation, it is
5   preferred that the molar ratio of GnSCN to lactam be about 1 to
3, and that the concentration of lactam to phenol range from
about 1:1 to about 1:5 or to about 5:1. The lactam of
preference for this procedure is N-methyl-2-pyrrolidone. See
Example VIII for further illustration.
10

Selective Extraction of Plasmid DNA.

Plasmid DNA may also be selectively isolated from
complex biological samples using the extraction methods of the
present invention. For example, methods to partially
15   solubilize cells so that chromosomal DNA is retained in the
cell and plasmid DNA is leaked into solution may be used. Weak
chaotropic salt solutions such as GnHCl, guanidine sulfate, or
guanidine carbonate, containing non-ionic detergents, may be
used to cause cells, particularly bacterial cells, to leak
20   plasmid DNA. The weak chaotropic salt solution will preferably
contain the chaotropic agent at a molar concentration of about
0.1 to 2M. The cells containing the chromosomal DNA may then
be removed from the solution containing the plasmid DNA, such
as by centrifugation, filtration or dialysis. The plasmid DNA
25   may then be extracted from the solution by any methods known in
the art or preferably by the extraction methods disclosed
herein. See Example IX for further illustration. It will be
obvious to those skilled in the art that other methods for
isolating plasmid DNA may be used in conjunction with the
30   extraction solutions disclosed herein, such as those found in
Methods in Molecular Biology, Vol. II, "Nucleic Acids," Humana
Press, Clifton, N.J. (1984) p.177-189, which is incorporated by
reference herein.

35   Extraction to Improve Hybridization.

It may also be advantageous to use the extraction
methods and solutions of this invention prior to conducting a
hybridization assay on a complex biological sample such as on

feces or blood. The extraction procedure may be necessary in
some cases to remove contaminants which contribute to
background interference. Extraction followed by procedures to
concentrate the nucleic acid may improve sensitivity and the
signal to noise ratio in hybridization assays. Hybridization
assays of particular interest include those set forth in
commonly assigned U.S. Application No. 07/384,235, filed
July 24, 1989.

          Conducting a hybridization assay according to the
present invention can be performed by any method known to those
skilled in the art or analogous to immunoassay methodology
given the guidelines presented herein. Preferred methods of
assay are the sandwich assays and variations thereof and the
competition or displacement assay. Hybridization techniques
are generally described in "Nucleic Acid Hybridization, A
Practical Approach," Ed. Hames, B.D. and Higgins, S.J., IRL
Press, 1985; Gall and Pardue (1969), Proc. Natl. Acad. Sci.,
U.S.A., 63:378-383; and John, Burnsteil and Jones (1969)
Nature, 223:582-587. As improvements are made in hybridization
techniques, they can readily be applied.

          Sandwich assays are commercially useful hybridization
assays for detecting or isolating nucleic acid sequences. Such
assays utilize a "capture" nucleic acid covalently immobilized
to a solid support and labelled "signal" nucleic acid in
solution. The clinical sample will provide the target nucleic
acid. The "capture" nucleic acid and "signal" nucleic acid
probe hybridize with the target nucleic acid to form a
"sandwich" hybridization complex. To be effective, the signal
nucleic acid cannot hybridize with the capture nucleic acid.

          In a hybridization assay, the target nucleic acid is
the nucleotide sequence of deoxyribonucleic acid (DNA),
ribonucleic acid (RNA) or ribosomal ribonucleic acid (rRNA)
whose presence is of interest and whose presence or absence is
to be detected for. The target nucleic acid may be provided in
a complex biological mixture of nucleic acid (RNA, DNA and/or
rRNA) and non-nucleic acid.

          The target nucleic acids of primary preference are
RNA molecules and, in particular, open regions of rRNA which

have minimal secondary or tertiary interactions with adjacent
nucleotides, such as on the 16s or 23s rRNA as described in
commonly assigned U.S. Patent Application No. 142,106, which is
incorporated by reference herein. If target nucleic acids of
5   choice are double stranded or otherwise have significant
secondary and tertiary structure, they may need to be heated
prior to hybridization. In this case, heating may occur prior
to or after the introduction of the nucleic acids into the
hybridization medium.
10   A nucleic acid substantially complementary to the
target nucleic acid will be introduced in the hybridization
process. "A nucleic acid substantially complementary to the
target nucleic acid" is a polynucleotide or oligonucleotide
containing naturally occurring nucleotides or their analogs,
15   such as 7-deazaguanosine or inosine, sufficiently complementary
to hybridize with the target nucleic acid such that stable and
specific binding occurs between the target and the
complementary nucleic acid. Therefore, the complementary
nucleic acid sequence need not reflect the exact sequence of
20   the target nucleic acid. For example, a non-complementary
nucleotide fragment may be attached to a complementary
nucleotide fragment or alternatively, non-complementary bases
or longer sequences can be interspersed into the complementary
nucleic acid, provided that the complementary nucleic acid
25   sequence has sufficient complementarity with the sequence of
the target nucleic acid to hybridize therewith forming a
hybridization complex and further is capable of immobilizing
the target nucleic acid to a solid support. The degree of
homology required for formation of a stable hybridization
30   complex (duplex) varies with the stringency of the
hybridization medium and/or wash medium. Oligonucleotide
probes to rRNA are most preferred such as those described in
commonly assigned U.S. Patent Application Serial No. 142,106,
which is incorporated by reference herein. The complementary
35   nucleic acid may be present in a pre-prepared hybridization
media or introduced at some later point prior to hybridization.
          The hybridization media and processes disclosed
herein, however, are uniquely suited to a one-step assay. The

media may be pre-prepared, either commercially or in the
laboratory to contain all the necessary components for
hybridization. For instance, in a sandwich assay the media
could comprise a lactam, desired buffers and detergents, a
5   capture nucleic acid bound to a solid support such as a
microbead, and a signal nucleic acid. This media then need
only be combined with the sample containing the target nucleic
acid at the time the assay is to be performed. Once
hybridization occurs the hybridization complex attached to the
10   solid support may be washed and the extent of hybridization
determined.

Virtually any solid surface can be used as a support
for hybridization assays, including metals and plastics. Two
types of solid surfaces are generally available namely:
15   a)   Membranes, polystyrene beads, nylon, teflon,
polystyrene/latex beads, latex beads or any solid support
possessing an activated carboxylate, sulfonate, phosphate or
similar activatable group are suitable for use as solid surface
substratum to which nucleic acids or oligonucleotides can be
20   immobilized.

b)   Porous membranes possessing pre-activated
surfaces which may be obtained commercially (e.g., Pall
Immunodyne Immunoaffinity Membrane, Pall BioSupport Division,
East Hills, NY, or Immobilon Affinity membranes from Millipore,
25   Bedford, Mass.) and which may be used to immobilize capture
oligonucleotides. Microbeads, including magnetic beads, of
polystyrene, teflon, silica, nylon or latex are particularly
useful.

Capture or signal nucleic acids for use in
30   hybridization assays can be obtained from the entire sequence
or portions thereof of an organism's genome, from messenger
RNA, or from cDNA obtained by reverse transcription of
messenger RNA. After isolation of genomic DNA or cDNA
fragments, the fragments are typically inserted into a
35   replication vector, such as lambda phage, pBR322, M13, or
vectors containing the SP6 or T7 promoter and cloned as a li-
brary in a bacterial host. Following appropriate screening
procedures, a recombinant vector with the desired probe insert

is isolated and labelled as described below. The vector is
then grown in a suitable host. The probe and its vector are
purified from the host cells by cell lysis and nucleic acid
extraction. Following isolation, the probe can be purified
5   away from the vector by digestion with selected restriction en-
zymes and sequenced. Further isolation of the probe can be
achieved by using gel electrophoresis or high pressure liquid
chromatography.

Once the appropriate sequences are determined, DNA
10   probes are preferably chemically synthesized using commercially
available methods and equipment. For example, the solid phase
phosphoramidite method can be used to produce short probes of
between 15 and 50 bases and have a molecular weight of less
than 16,000 daltons. (Caruthers et al., Cold Spring Harbour
15   Symp. Quant. Biol., 47:411-418 (1982), and Adams et al., J. Am.
Chem. Soc., 105:661 (1983).

When synthesizing a probe for a specific target, the
choice of nucleotide sequence will determine the specificity of
the test. For example, by comparing DNA sequences from several
20   virus isolates, one can select a sequence for virus detection
that is either type specific or genus specific. Comparisons of
DNA regions and sequences can be achieved using commercially
available computer programs.

The determination of the extent of hybridization may
25   be done by any of the methods well-known in the art. If there
is no detectable hybridization, the extent of hybridization is
thus 0. Typically labelled signal nucleic acids are used to
detect hybridization. Complementary nucleic acids or signal
nucleic acids may be labelled by any one of several methods
30   typically used to detect the presence of hybridized
polynucleotides. The most common method of detection is the
use of autoradiography with ^{3}H, ^{125}I, ^{35}S, ^{14}C, or ^{32}P labelled
probes or the like. The choice of radioactive isotope depends
on research preferences due to ease of synthesis, varying
35   stability, and half lives of the selected isotopes. Other
labels include ligands which bind to labelled antibodies,
fluorophores, chemiluminescent agents, enzymes, and antibodies
which can serve as specific binding pair members for a labelled

ligand. The choice of label depends on sensitivity required,
ease of conjugation with the probe, stability requirements, and
available instrumentation.

5   The choice of label dictates the manner in which the
label is bound to the probe. Radioactive probes are typically
made using commercially available nucleotides containing the
desired radioactive isotope. The radioactive nucleotides can
be incorporated into probes by several means such as by nick
translation of double-stranded probes; by copying single-
10 stranded M13 plasmids having specific inserts with the Klenow
fragment of DNA polymerase in the presence of radioactive dNTP;
by transcribing cDNA from RNA templates using reverse trans-
criptase in the presence of radioactive dNTP; by transcribing
RNA from vectors containing SP6 promoters or T7 promoters using
15 SP6 or T7 RNA polymerase in the presence of radioactive rNTP;
by tailing the 3' ends of probes with radioactive nucleotides
using terminal transferase; or by phosphorylation of the 5'
ends of probes using $[^{32}P]$-ATP and polynucleotide kinase.

           Non-radioactive probes are often labelled by indirect
20 means. Generally, a ligand molecule is covalently bound to the
probe. The ligand then binds to an anti-ligand molecule which
is either inherently detectable or covalently bound to a signal
system, such as a detectable enzyme, a fluorescent compound, or
a chemiluminescent compound. Ligands and anti-ligands may be
25 varied widely. Where a ligand has a natural anti-ligand, for
example, biotin, thyroxine, and cortisol, it can be used in
conjunction with the labelled, naturally occurring anti-
ligands. Alternatively, any haptenic or antigenic compound can
be used in combination with an antibody.

30   Probes can also be conjugated directly to signal
generating compounds, e.g., by conjugation with an enzyme or
fluorophore. Enzymes of interest as labels will primarily be
hydrolases, particularly phosphatases, esterases and
glycosidases, or oxidoreductases, particularly peroxidases.
35 Fluorescent compounds include fluorescein and its derivatives,
rhodamine and its derivatives, dansyl, umbelliferone, etc.
Chemiluminescent compounds include luciferin, AMPPD ([3-(2'-

spiroamantane)-4-methoxy-4-(3'phosphoryloxy)-phenyl-1,2-
dioxetane]) and 2,3-dihydrophthalazinediones, e.g., luminol.

    The amount of labelled probe which is present in the
hybridization medium or extraction solution may vary widely.
5   Generally, substantial excesses of probe over the
stoichiometric amount of the target nucleic acid will be
employed to enhance the rate of binding of the probe to the
target DNA.   Treatment with ultrasound by immersion of the
reaction vessel into commercially available sonication baths
10 can often times accelerate the hybridization rates.

    After hybridization at a temperature and time period
appropriate for the particular hybridization solution used, the
glass, plastic, or filter support to which the capture nucleic
acid-target nucleic acid hybridization complex is attached is
15 introduced into a wash solution typically containing similar
reagents (e.g., sodium chloride, buffers, organic solvents and
detergent), as provided in the hybridization solution.   These
reagents may be at similar concentrations as the hybridization
medium, but often they are at lower concentrations when more
20 stringent washing conditions are desired.   The time period for
which the support is maintained in the wash solutions may vary
from minutes to several hours or more.

    Either the hybridization or the wash medium can be
stringent.   After appropriate stringent washing, the correct
25 hybridization complex may now be detected in accordance with
the nature of the label.

    The probe may be conjugated directly with the label.
For example, where the label is radioactive, the probe with
associated hybridization complex substrate is exposed to X-ray
30 film.   Where the label is fluorescent, the sample is detected
by first irradiating it with light of a particular wavelength.
The sample absorbs this light and then emits light of a
different wavelength which is picked up by a detector (Physical
Biochemistry, Freifelder, D., W.H. Freeman & Co. (1982), pp.
35 537-542).   Where the label is an enzyme, the sample is detected
by incubation on an appropriate substrate for the enzyme.   The
signal generated may be a colored precipitate, a colored or
fluorescent soluble material, or photons generated by

bioluminescence or chemi-luminescence. The preferred label for probe assays generates a colored precipitate to indicate a positive reading. For example, alkaline phosphatase will dephosphorylate indoxyl phosphate which then will participate

5   in a reduction reaction to convert tetrazolium salts to highly colored and insoluble formazans.

Detection of a hybridization complex may require the binding of a signal generating complex to a duplex of target and probe polynucleotides or nucleic acids. Typically, such

10   binding occurs through ligand and anti-ligand interactions as between a ligand-conjugated probe and an anti-ligand conjugated with a signal. The binding of the signal generation complex is also readily amenable to accelerations by exposure to ultrasonic energy.

15   The label may also allow indirect detection of the hybridization complex. For example, where the label is a hapten or antigen, the sample can be detected by using antibodies. In these systems, a signal is generated by attaching fluorescent or enzyme molecules to the antibodies or

20   in some cases, by attachment to a radioactive label. (Tijssen, P., "Practice and Theory of Enzyme Immunoassays," Laboratory Techniques in Biochemistry and Molecular Biology, Burdon, R.H., van Knippenberg, P.H., Eds., Elsevier (1985), pp. 9-20.)

Kits for the extraction of and hybridization of

25   nucleic acids are also contemplated. Such kits would contain at least one vial containing an extraction solution or a hybridization medium which comprises a lactam at a concentration in excess of 5%. Detergents, buffer solutions and additional vials which contain components to detect target

30   nucleic acids may also be included.

The following examples are offered by way of illustration and are not to be construed as limiting the invention, as claimed, in any way.

EXAMPLE I: Lysis of bacterial and human cells with

35   pyrrolidone-based solutions.

A pre-prepared lysis solution composed of 20% N-cyclohexyl-2-pyrrolidone, and 20% N-hydroxymethyl-2-pyrrolidone, (both obtained from GAF Chemicals Corporation,

Wayne, New Jersey), 50 mM Tris, pH 7.6, 25 mM EDTA, and 2% SDS
(sodium dodecyl sulfate) was used to lyse 1×10^9 cells of
Actinobacillus actinomycetecomitans ("Aa"), Bacteroides
intermedius ("Bi"), Eikenella corrodens ("Ec"), Wolinella recta
5   ("Wr"), Fusobacterium nucleatum ("Fn"), Bacteroides gingivalis
("Bg"), and 1×10^6 cells of HeLa in 100 microliter volumes
each. The solution was noted to clear within 10 seconds and
phase contrast microscopy indicated the solubilization of all
the cell types tested. No intact cell shapes were observed by
10 light microscopy. In addition, the lysates were observed by
fluorescence microscopy in which ethidium bromide was added to
the lysate at a concentration of 0.5 micrograms per ml. Only
extremely diffuse staining was observed in all lysates compared
to intact cell controls, indicating that lysis was complete and
15 that chromosomal DNA was unfolded and released from the cells.
            EXAMPLE II: Lysis of bacterial and human cells with
pyrrolidone-based solutions containing a chaotropic agent.
            A lysis solution composed of 20% N-cyclohexyl-2-
pyrrolidone, 20% N-hydroxymethyl-2-pyrrolidone, 10% N-dodecyl-
20 2-pyrrolidone, 1M Guanidine-HCl, 50 mM Tris, pH 7.6, 25 mM
EDTA, and 2% SDS was used to lyse 1×10^9 cells of Aa, Bi, Ec,
Wr, Fn, Bg, and 1 x 10^6 cells of HeLa in 100 microliter
volumes. The solution was noted to clear within 10 seconds and
phase contrast microscopy indicated the solubilization of all
25 the cell types tested. No intact cell shapes were observed by
light microscopy. In addition, the lysates were observed by
fluorescence microscopy in which ethidium bromide was added to
the lysate at a concentration of 0.5 micrograms per ml. Only
extremely diffuse staining was observed in all lysates compared
30 to intact cell controls, indicating that lysis was complete and
that chromosomal DNA was unfolded and released from the cells.
            EXAMPLE III: Combination pyrrolidone-based
hybridization solutions promote specific nucleic acid base
pairing.
35   A solution composed of 20% N-cyclohexyl-2-
pyrrolidone, 20% N-hydroxymethyl-2-pyrrolidone, 50 mM Tris pH
7.6, 25 mM EDTA and 1% SDS was used to test the ability of ^{32}P-
labelled specific oligonucleotide probes (synthetic

oligonucleotide probes in which the sequence is complementary
to unique hypervariable regions of the 16s rRNA of the
respective bacterium) to hybridize specifically to total
nucleic acid from Aa, Bg, Bi, Ec, Fn, Wr and E. coli

5   immobilized on Nytran filters (Schleicher and Schuell, Keene,
New Hampshire). Approximately 50 nanograms of total nucleic
acid was immobilized on each nytran slot. 6.5×10^6 cpm of ^{32}P
labelled probe (approximately 65 nanograms) of Aa complementary
probe (Aa4B) was added to 1ml of the solution described above

10   and incubated with nytran filter strips containing Aa, Bg, Bi,
Ec, Fn, Wr, and E. coli slots of nucleic acid. Hybridization
was allowed to proceed for 3 hours at 19°C. The filters were
washed with 0.09M NaCl, 50 mM Tris pH 7.6, 25 mM EDTA and 0.1%
SDS at 50°C and radioactivity was then detected by fluorometry.

15   Results indicated that the Aa probes hybridized only with Aa
total nucleic acid, demonstrating that the pyrrolidone solution
promoted specific nucleic acid base pairing. Identical results
were obtained using solutions of the same composition described
above with the exception of altering each pyrrolidone

20   concentration to 15% or 25% respectively.

        EXAMPLE IV: Individual pyrrolidone-based
hybridization solutions promote specific nucleic acid base
pairing.

        A solution composed of 40% N-cyclohexyl-2-

25   pyrrolidone, or 40% N-hydroxymethyl-2-pyrrolidone, or 40% N-
methyl-2-pyrrolidone, 50 mM Tris pH 7.6, 25 mM EDTA and 1% SDS
was used to test the ability of ^{32}P-labelled specific
oligonucleotide probes (synthetic oligonucleotide probes in
which the sequence is complementary to unique hypervariable

30   regions of the 16s rRNA of the respective bacterium) to
hybridize specifically to total nucleic acid from Aa, Bg, Bi,
Ec, Fn, Wr, and E. coli immobilized on Nytran filters.
Approximately 50 nanograms of total nucleic acid was
immobilized on each nytran slot. 6.5×10^6 cpm of ^{32}P-labelled

35   probe (approximately 65 nanograms) of Aa complementary probe
(Aa4B) was added to 1ml of the solution described above and
incubated with nytran filter strips containing Aa, Bg, Bi, Ec,
Fn, Wr, and E. coli slots of nucleic acid. Hybridization was

allowed to proceed for 3 hours at 19°C. The filters were
washed with 0.09M NaCl, 50 mM Tris pH 7.6, 25 mM EDTA and 0.1%
SDS (SDS/FW) at 50°C and radioactivity was then detected by
fluorometry. Results indicated that the Aa probes hybridized
5   only with Aa total nucleic acid demonstrating that the
individual pyrrolidone solutions promoted specific nucleic acid
base pairing. Identical results were obtained using solutions
of the same composition described above with the exception of
altering the individual pyrrolidone concentration to 30% or
10   50%, respectively.
         EXAMPLE V: Individual pyrrolidone-based
hybridization solutions containing chaotropic agents,
piperidone-based hybridization solutions and caprolactam or
valerolactam-based hybridization solutions promote specific
15   nucleic acid base pairing.
         A solution composed of 35% N-methyl-2-piperidone, or
35% 2-ε-caprolactam, or 35% N-methyl-2-caprolactam, or 35% 2-
δ-valerolactam, 50 mM Tris pH 7.6, 25 mM EDTA and 1% SDS or a
solution containing 20% N-cyclohexyl-2-pyrrolidone, 20% N-
20   hydroxyethyl-2-pyrrolidone, 5% N-dodecyl-2- pyrrolidone, 1M
guanidine-HCl, 50 mM Tris pH 7.6, 25 mM EDTA and 2% SDS, or a
solution containing 20% N-cyclohexyl-2- pyrrolidone, 20% N-
hydroxyethyl-2-pyrrolidone, 5% N-dodecyl-2-pyrrolidone, 0.5M
guanidine HCl, 50 mM Tris pH 7.6, 25 mM EDTA and 2% SDS, was
25   used to test the ability of ^{32}P-labelled specific
oligonucleotide probes (synthetic oligonucleotide probes in
which the sequence is complementary to unique hypervariable
regions of the 16s rRNA of the respective bacterium) to
hybridize specifically to total nucleic acid from Aa, Bg, Bi,
30   Ec, Fn, Wr, and E. coli immobilized on Nytran filters.
Approximately 50 nanograms of total nucleic acid was
immobilized on each nytran slot. 6.5 x 10^6 cpm of P32-labelled
probe (approximately 65 nanograms) of Bg complementary probe
(Bg5B) was added to 1ml of the solution described above and
35   incubated with nytran filter strips containing Aa, Bg, Bi, Ec,
Fn, Wr, and E. coli slots of nucleic acid. Hybridization was
allowed to proceed for 3 hours at 19°C. The filters were
washed with 0.09 M NaCl, 50 mM Tris pH 7.6, 25 mM EDTA and 0.1%

SDS (SDS/FW) at 19°C and radioactivity was then detected by
fluorometry. Results indicated that the Bg probes hybridized
only with the Bg total nucleic acid demonstrating that the
individual piperidone or lactam-based hybridization solutions
5   promoted specific nucleic acid base pairing.

EXAMPLE VI: Specific detection of Bg bacterium in
pyrrolidone-based hybridization media.

A hybridization media composed of 20% N-cyclohexyl-
2-pyrrolidone, and 20% N-hydroxymethyl-2-pyrrolidone, 50 mM
10   Tris, pH 7.6, 25 mM EDTA, and 2% SDS was used to lyse 1 x 10^8
cells of Aa, Bi, Ec, Wr, Fn, or Bg in 100 microliter volumes at
19°C. Biotinylated 24-mer oligonucleotide probes complementary
to conserved regions of bacterial 16s rRNA (target probes) were
added to a final concentration of 100 nanograms per ml. This
15   solution was then incubated with nytran discs which had
covalently immobilized 1 microgram of Bg specific
oligonucleotide probe (capture probe) for 1 hour at ambient
temperature. The solid supports were then washed with SDS/FW
at ambient temperature and then incubated with 10ng/ml of
20   Strepavidin/Horseradish peroxidase (SA/HRP) conjugate in SDS/FW
for 30 minutes at ambient temperature. The solid supports were
then washed with SDS/FW, FW (filtered water), and then the
presence of peroxidase was determined by incubating the filter
with a substrate that formed an insoluble product. The results
25   indicated that only the Bg bacterium was detected in the
colorimetric sandwich assay. The pyrrolidone hybridization
media therefore promoted effective lysis and specific nucleic
acid base pairing of the target nucleic acid.

EXAMPLE VII: Extraction of total nucleic acid or
30   DNA.

A.   Extraction of total nucleic acid or DNA from
bacterial cells using GnSCN, heat and a lactam.

To recover total nucleic acid from GnSCN-solubilized
bacterial cells approximately 1 x 10^9 Bg cells were lysed in
35   100 microliters of 3M GnSCN, 50 mM Tris pH 7.6, 25 mM EDTA and
2% Sarkosyl. An equivalent of N-methyl-2-pyrrolidone was added
(100 microliters) followed by 500 microliters of phenol and 300
microliters of 1% SDS, 50 mM Tris pH 7.6, 25 mM EDTA and 0.05M

NaCl. The solution was then heated to 65°C for 10 minutes and
then mixed for 3 minutes at ambient temperature. The solution
was then subjected to centrifugation at 10,000 rpm for 5
minutes to force phase separation. The upper aqueous phase was

5   then decanted and nucleic acid was precipitated with 2 volumes
of 100% ethanol. Nucleic acid was pelleted for 5 minutes at
10,000 rpm at ambient temperature and the resulting pellet was
solubilized with 100 microliters of water. An aliquot was
examined by gel electrophoresis. The results indicate that

10 chromosomal DNA and rRNA were efficiently extracted from the
GnSCN solubilized bacterial cells.

B. Extraction of total nucleic acid or DNA from
bacterial cells using GnSCN and ethylene glycol without the use
of a heating step.

15   To recover total nucleic acid from GnSCN-solubilized
bacterial cells, approximately 1×10^8 Bg cells were lysed in 100
microliters of 3M GnSCN, 50 mM Tris pH 7.6, 25 mM EDTA and 2%
Sarkosyl. 100 microliters of ethylene glycol was then added to
the lysate followed by 500 microliters of phenol and 300

20 microliters of 1% SDS, 50 mM Tris pH 7.6, 25 mM EDTA and 0.05M
NaCl. The solution was mixed for 1-3 minutes at ambient
temperature. The solution was then subjected to centrifugation
at 10,000 rpm for 5 minutes to force phase separation. The
upper aqueous phase was then decanted and nucleic acid was

25 precipitated with 2 volumes of 100% ethanol. Nucleic acid was
pelleted for 5 minutes at 10,000 rpm at ambient temperature and
the resulting pellet was solubilized with 100 microliters of
water. An aliquot was examined by gel electrophoresis. The
results indicate that chromosomal DNA and rRNA were efficiently

30 extracted from the GnSCN solubilized bacterial cells.

C. Extraction of total nucleic acid or DNA from
bacterial cells using GnHCl and a lactam without the use of a
heating step.

To recover total nucleic acid from GnHCl-solubilized

35 bacterial cells approximately 1×10^9 Bg cells were lysed in 100
microliters of 3M GnHCl, 4M urea, 40 mM Tris pH 7.6, 25 mM EDTA
and 2% Sarkosyl. 100 microliters of N-methyl-2-pyrrolidone was
added to the solution followed by 500 microliters of phenol and

300 microliters of 1% SDS, 50 mM Tris pH 7.6, 25 mM EDTA and
0.05M NaCl. The solution was mixed for 3 minutes at ambient
temperature. The solution was then subjected to centrifugation
at 10,000 rpm for 5 minutes to force phase separation. The
5   upper aqueous phase was then decanted and nucleic acid was
precipitated with 2 volumes of 100% ethanol. Nucleic acid was
pelleted for 5 minutes at 10,000 rpm at ambient temperature and
the resulting pellet was solubilized with 100 microliters of
water. An aliquot was examined by gel electrophoresis. The
10   results indicate that total nucleic acid was efficiently
extracted from the GnHCl solubilized bacterial cells.
        EXAMPLE VIII: The sequential isolation of DNA and
rRNA from bacterial cell solubilized with 3M GnSCN.
        To recover DNA and rRNA sequentially from GnSCN-
15   solubilized bacterial cells approximately 1×10^9 Bg cells were
lysed in 100 microliters of 3M GnSCN, 50 mM Tris pH 7.6, 25 mM
EDTA and 2% Sarkosyl. An equivalent of N-methyl-2-pyrrolidone
was added (100 microliters) followed by 500 microliters of
phenol and 300 microliters of 1% SDS, 50 mM Tris pH 7.6, 25 mM
20   EDTA and 0.05M NaCl. The solution was mixed for 3 minutes at
ambient temperature and then subjected to centrifugation at
10,000 rpm for 5 minutes to force phase separation. The upper
aqueous phase was then decanted and nucleic acid was
precipitated with 2 volumes of 100% ethanol. Another 300
25   microliters of 1% SDS, 50 mM Tris, 25 mM EDTA and 0.05M NaCl
was added to the organic (lower phase) and the solution was
then heated to 65°C for 10 minutes and then mixed for 3 minutes
at ambient temperature. Again, the nucleic acid in the upper
aqueous phase was precipitated by the addition of two volumes
30   of 100% ethanol. Nucleic acid was pelleted for 5 minutes at
10,000 rpm at ambient temperature and the resulting pellet was
solubilized with 100 microliters of water. An aliquot was
examined by gel electrophoresis. The results indicate that
first chromosomal DNA was recovered from the GnSCN lysate
35   followed by the recovery of rRNA. Both nucleic acid types were
efficiently extracted and purified from the GnSCN solubilized
bacterial cells. This procedure permits the recovery and

separation of DNA and rRNA from cells solubilized with chaotropic salts such as GnSCN.

EXAMPLE IX: Recovery of plasmid DNA from bacterial cells partially solubilized with guanidine-HCl.

5   Bacterial cells such as E. coli can be partially solubilized with weak chaotropic salt solutions such as guanidine-HCl which contain non-ionic detergents. Partially solubilized cells leak RNA and plasmid DNA while retaining chromosomal DNA. These observations were used to develop a

10   method for the rapid and simple isolation of plasmid DNA from E. coli.

Approximately 1×10^9 E. coli cells transfected with PBR 322 plasmid were partially solubilized with 100 microliters of 2M guanidine-HCl, 50 mM Tris, 25 mM EDTA and 2% Sarkosyl.

15   The cells were incubated without agitation for 2 minutes at ambient temperature. After the incubation cells and insoluble material was pelleted at 10,000 rpm for 1 minute and the supernatant was decanted and reserved. To the supernatant 100 microliters of N-methyl-2-pyrrolidone was added followed by 500

20   microliters of phenol and 300 microliters of SDS-extraction buffer.

The solution was mixed for 3 minutes at ambient temperature and the solution was then subjected to centrifugation at 10,000 rpm for 5 minutes to force phase

25   separation. The upper aqueous phase was then decanted and nucleic acid was precipitated with 2 volumes of 100% ethanol. Nucleic acid was pelleted for 5 minutes at 10,000 rpm at ambient temperature and the resulting pellet was solubilized with 100 microliters of water. An aliquot was examined by gel

30   electrophoresis. The results indicate that relative to total extracted nucleic acid, plasmid DNA was preferentially extracted, essentially free of chromosomal DNA, rRNA and protein.

EXAMPLE X: Extraction and immobilization of total

35   nucleic acid from bacterial cells onto solid supports for the detection of pathogens in complex biological samples using oligonucleotide probes.

To recover total nucleic acid from GnSCN-solubilized
bacterial cells a combination of approximately 1×10^8 Aa, Bg,
Bi, Ek, Fn, Wr cells were lysed in 100 microliters of 3M GnSCN,
50 mM Tris pH 7.6, 25 mM EDTA and 2% Sarkosyl. An equivalent
5   of N-methyl-2-pyrrolidone was added (100 microliters) followed
by 500 microliters of phenol and 300 microliters of 1% SDS, 50
mM Tris pH 7.6, 25 mM EDTA and 0.05M NaCl. The solution was
then heated to 65°C for 10 minutes and then mixed for 3 minutes
at ambient temperature. The solution was then subjected to
10   centrifugation at 10,000 rpm for 5 minutes to force phase
separation. 20 microliter portions of the upper aqueous phase
were then slotted into Nytran filters using a slotting
apparatus.

The filters were then heated to 95°C for 3 hours.
15   Approximately 50 nanograms of total nucleic acid was
immobilized on each nytran slot. 6.5×10^6 cpm of ^{32}P-labelled
probe (approximately 65 nanograms) of either Aa, Bg, Bi, Ek,
Fn, or Wr complementary probe was added to 1ml of the
pyrrolidone-based hybridization solution described above and
20   incubated with nytran filter strips containing Aa, Bg, Bi, Ec,
Fn, Wr, and E. coli slots of nucleic acid. Hybridization was
allowed to proceed for 3 hours at 19°C. The filters were
washed with 0.09M NaCl, 50 mM Tris pH 7.6, 25 mM EDTA and 0.1%
SDS (SDS/FW) at ambient temperature and radioactivity was then
25   detected by fluorometry. Results indicated that the specific
probes hybridized only with their respective total nucleic acid
indicating that target nucleic acid can be directly immobilized
onto a solid support after extraction of chaotropic salts
containing solubilized nucleic acid with lactam-based
30   solutions.

EXAMPLE XI: The use of lactams to isolate total
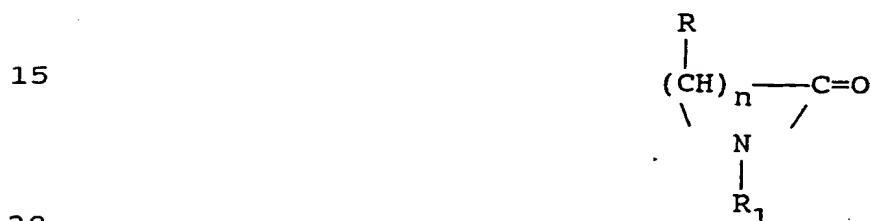nucleic acid from bacterial cells without the use of phenol.

A pre-prepared lysis solution composed of 20% N-
cyclohexyl-2-pyrrolidone, 20% N-hydroxymethyl-2-pyrrolidone,
35   10% N-dodecyl-2-pyrrolidone, 50 mM Tris, pH 7.6, 25 mM EDTA,
and 2% SDS was used to lyse 1×10^9 cells of Aa, Bi, Ec, Wr,
Fn, Bg, and 1×10^6 cells of HeLa in 100 microliter volumes.
An equal volume of N-methyl-2-pyrrolidone was then added

followed by the addition of 200 microliters of N-dodecyl-2-
pyrrolidone. The solution immediately became biphasic.

The solution was mixed for 3 minutes at ambient
temperature and the solution was then subjected to
5   centrifugation at 10,000 rpm for 5 minutes to force phase
separation. The upper aqueous phase was then decanted and
nucleic acid was precipitated with 2 volumes of 100% ethanol.
Nucleic acid was pelleted for 5 minutes at 10,000 rpm at
ambient temperature and the resulting pellet was solubilized
10   with 100 microliters of water. An aliquot was examined by gel
electrophoresis. The results indicate that total nucleic acid
was preferentially extracted and purified from the cell lysate.

Example XII: One step assay to detect specific
nucleic acid sequences of bacterial pathogens.
15   A pre-prepared lysis solution composed of 20% N-
cyclohexyl-2-pyrrolidone, 20% N-hydroxymethyl-2-pyrrolidone,
10% N-dodecyl-2-pyrrolidone, 50 mM Tris, pH 7.6, 25 mM EDTA,
and 2% SDS and containing 1 to 5 mg of 5 micron beads (silica,
(Spherisorb) from Phase Sep, Deeside Ind., Queensferry, Clwyd,
20   U.K.) onto which 1 to 2 micrograms of Bg specific
oligonucleotide probe has been covalently immobilized, and
which also contained 1×10^6 cpm of ^{32}P oligonucleotide probe
(specific activity of 1×10^7 cpm per microgram) complementary
to Bg specific regions of the 16s rRNA was used to lyse 1×10^6
25   cells of Aa, Bi, Ec, Wr, Fn, and Bg in 100 microliter volumes
at 19°C. The solution was then incubated for 30 minutes at room
temperature. The solid supports were then washed with SDS/FW
at ambient temperature to remove un-hybridized material and
radioactive probes. The solid supports were then monitored for
30   radioactivity by scintillation counting. The results indicated
that only Bg cells were detected when using Bg specific
oligonucleotide signal probes and not when using specific
labelled probes for Aa, Bi, Ek, Fn or Wr. Thus, in 30 minutes 1
$\times 10^6$ Bg cells were detected in a simple one step hybridization
35   assay.

WHAT IS CLAIMED IS:

1.   A method for isolating nucleic acid from a
sample containing a complex biological mixture of nucleic acids
5   and non-nucleic acids said method comprising combining the
sample with an extraction solution comprising at least one
lactam and then isolating nucleic acid from the resulting
combined solution.

10   2.   The method of claim 1, wherein the lactam is
selected from the group of compounds having the formula:

$$
\begin{array}{c}
R \\
| \\
(CH)_n\!\!-\!\!C=O \\
\diagdown \qquad \diagup \\
N \\
| \\
R_1
\end{array}
$$

20

wherein n = 3 to 8 (with the total number of R substituents
being 3 to 8), and R_1 is selected from the group consisting of:

25   hydrogen,
            alkyl of 1 to 20 carbons,
            aryl of 6 to 10 carbons,
            arylalkyl of 7 to 20 carbons,
            alkylcarboxyamide of 1 to 20 carbons,
30   hydroxyarylalkyl of 7 to 20 carbons,
            N-mono-substituted alkylcarboxyamide,
            N, N-dialkyl-substituted alkylcarboxyamide,
            alkylcarboxylate of 2 to 20 carbons,
            acyl of 2 to 20 carbons,
35   cycloalkane of 4 to 10 carbons,
            hydroxyalkyl,
            and cyanoalkyl;
            and where the R substituents can be the same or
different and selected from a group consisting of hydrogen,
40   halogen and alkyl of 1 to 5 carbons such that the total carbon
number for all R substituents does not exceed 20;

with the further provision that the total carbon number of the R_1 substituent does not exceed 20 and where at least one R designated substituent must be hydrogen.

3.   The method of claim 2, wherein n = 3 to 6.

4.   The method of claim 2, wherein at least one R substituent is methyl.

5.   The method of claim 2, wherein the R_1 substituent and all of the R substituents are hydrogen.

6.   The method of claim 2, wherein the R_1 substituent is other than hydrogen and all of the R substituents are hydrogen.

7.   The method of claim 2, wherein the R_1 substituent is a cycloalkane of 4 to 7 carbons.

8.   The method of claim 2, wherein the R_1 substituent is $-CH_2-CH_2OH$.

9.   The method of claim 2, wherein the R_1 substituent is hydroxyarylalkyl.

10.   The method of claim 2, wherein the R_1 substituent is dodecyl.

11.   The method of claim 2, wherein the R_1 substituent is methyl.

12.   The method of claim 1, wherein the lactam is a compound selected from the group consisting of 2-pyrrolidone, N-ethyl-2-pyrrolidone, N-cyclohexyl-2-pyrrolidone, N-dodecyl-2-pyrrolidone, N-methyl-2-pyrrolidone, N-hydroxyethyl-2-pyrrolidone, N-methyl-2-piperidone, 2-ε-caprolactam, N-methyl-2-caprolactam, 2-piperidone and N-(4-hydroxybenzyl)pyrrolidone.

13. The method of claim 1, wherein the resulting combined solution is further mixed and allowed to become biphasic before the nucleic acid material is isolated.

5   14. The method of claim 13, wherein the nucleic acid material is isolated by precipitation with ethanol from an aqueous phase.

15. The method of claim 1, wherein the resulting
10 combined solution further comprises a chaotropic agent.

16. The method of claim 1, wherein the sample is also combined with a solution comprising GnHCl.

15   17. The method of claim 1, wherein the lactam is about 5 to about 70% of the extraction solution.

18. A composition for the extraction of nucleic acid comprising at least one lactam at a concentration in excess of
20 at least about 5%, a detergent and a buffer solution.

19. A nucleic acid extraction kit comprising one vial containing an extraction solution which comprises a lactam at a concentration in excess of 5%.
25

20. A method for the selective extraction of nucleic acid from a complex biological sample comprising lysing the sample in a guanidine thiocyanate buffer; combining the sample with an extraction solution containing a lactam and phenol to
30 create a first aqueous phase and a first organic phase; and isolating DNA from the first aqueous phase.

21. The method of claim 20, wherein ribosomal RNA is further isolated from the first organic phase.
35

22. The method of claim 21, wherein the ribosomal RNA is isolated by adding a buffer solution to the first organic phase, heating the first organic phase and buffer

solution mixture to create a second organic phase and a second aqueous phase, and extracting rRNA from the aqueous phase.

23. A method for the selective extraction of plasmid DNA from a complex biological sample containing cells comprising partially solubilizing cells in the sample in a weak chaotropic salt solution, removing insoluble material from the solution and then extracting plasmid DNA from the solution.

24. The method of claim 23, wherein the weak chaotropic salt solution comprises guanidine hydrochloride.

25. A method for the extraction of nucleic acid from a sample containing a complex biological mixture of nucleic acid and non-nucleic acids without using heat said method comprising combining the sample with an extraction solution containing a guanidine salt, then adding a competitive agent selected from the group consisting of alcohol, polymeric sulfonic acid, and polysaccharide sulfate, rendering the solution biphasic and isolating the nucleic acid from an aqueous phase.

26. The method of claim 25 wherein the competitive agent is a dihydroxyalkane.

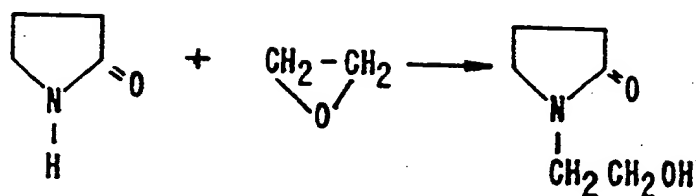27. The method of claim 25 wherein the competitive agent is ethylene glycol added at a molarity approximately comparable to or in excess of the guanidine salt.
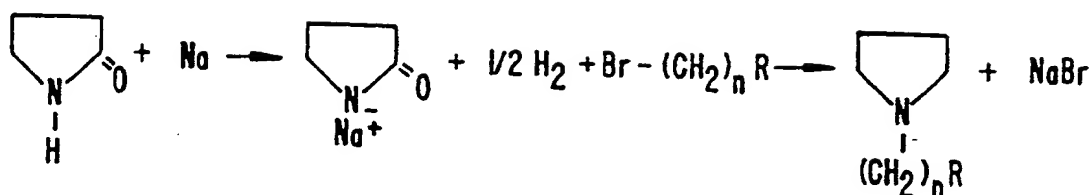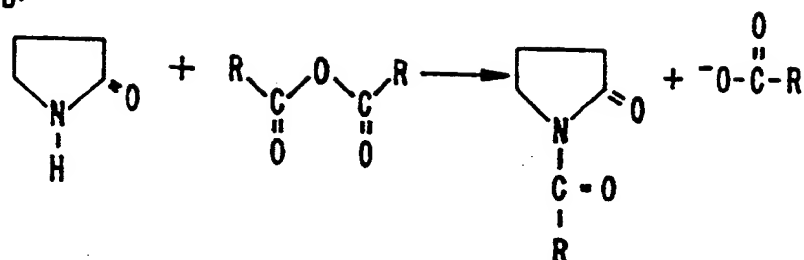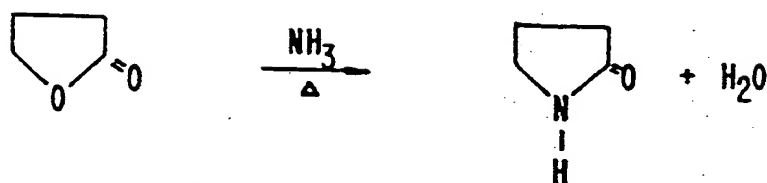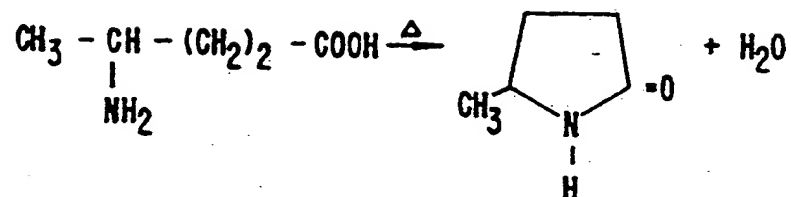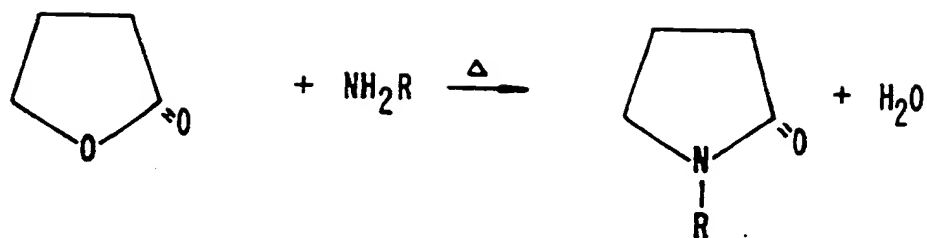
METHOD A:

METHOD B:

METHOD C:

METHOD D:

METHOD E:

$CH_3 - CH - (CH_2)_2 - COOH \xrightarrow{\Delta}$ ... $+ H_2O$

FIG._IA.

METHOD F:

$$+ \ NH_2R \ \xrightarrow{\Delta} \ \text{(pyrrolidinone)} \ + \ H_2O$$

METHOD G:

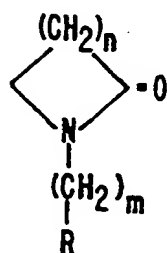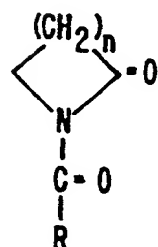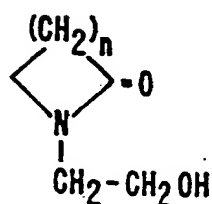$$\xrightarrow[\text{N}_3\text{H, H}^+]{\text{HYDRAZOIC ACID}} \ + \ N_2$$
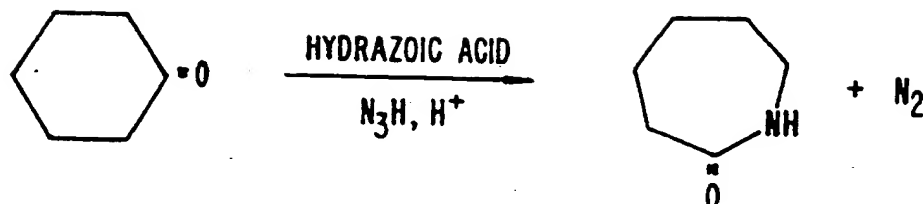
WHERE

m = 1 TO 20

IS PREPARED BY METHODS A, D

WHERE

IS PREPARED BY METHOD B

WHERE

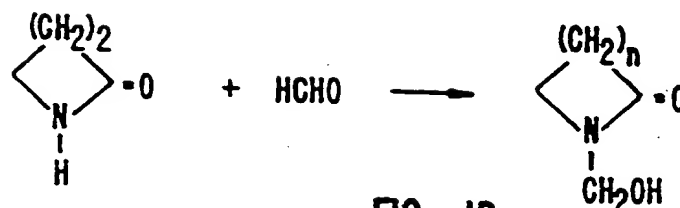IS PREPARED BY METHOD C

WHERE

IS PREPARED AS DESCRIBED:

$$+ \ HCHO \ \longrightarrow$$

FIG._IB.

INTERNATIONAL SEARCH REPORT

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ²

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5):C12Q 1/68;C07D 211/40,207 /40, ;225/02,223/10, 223/12;C12N 15/C

US. CL.: 548/543,546,547; 546/243; .540/451,463,485,526,527; 435/6

II. FIELDS SEARCHED

Minimum Documentation Searched ⁴	
Classification System ·	Classification Symbols
US CL.:	548/543,546; 546/243; 540/ .451,463,485,526; 548/547; 536/127; 540/527; 435/6

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched ⁸

III. DOCUMENTS CONSIDERED TO BE RELEVANT ¹⁴

Category *	Citation of Document, ¹⁶ with indication, where appropriate, of the relevant passages ¹⁷	Relevant to Claim No. ¹⁸
X Y	US, A, 4,483,920 (Gillespie et al) 20 November 1984, see abstract, col. 3-4.	23-27 / 20-22
X Y	EP, A, 0,127,327 (Ashley et al) 06 December 1984. See full document. especially pages 4-7.	23-27 / 1-22

* Special categories of cited documents: ¹⁵

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search ²	Date of Mailing of this International Search Report ⁴
30 November 1990	**17 JAN 1991**
International Searching Authority ¹	Signature of Authorized Officer ⁴
ISA/US	FRED TSING

Form PCT/ISA/210 (second sheet) (May 1988)

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE ¹

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers   because they relate to subject matter ¹ not required to be searched by this Authority, namely:

2. ☐ Claim numbers ...... , because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out ·, specifically:

3. ☐ Claim numbers_____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING²

This International Searching Authority found multiple inventions in this international application as follows:

I. Method of isolation of nucleic acids using lactams. Claim 1-14, 17-19.
II. Method of isolation of nucleic acids using lactams and chaotropic agents. Claims 15-16, 20-22.
III. Method of isolating nucleic acids using Chaotropic agents 23-27.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.
                          Telephone practice
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

Attachment to PCT\ISA\210

VI.   Observations Where Unity of Invention is Lacking.

I.   Claims 1-14, 17-19 drawn to methods for isolation of nuclei
      acids requiring lactams only.

II.   Claims 15-16 drawn to isolation of nucleic acids requirin
      lactams in combination with chaotropic agents.

III. Claims 23-27 drawn to isolation of nucleic acids requirin
      chaotropic agents only.

      The Claims of these three groups are directed to differen
inventions as the methods require distinct compositions fo
isolation or extraction.